PRODUCTION OF STAPHYLOCOCCUS STRAINS RESISTANT TO VARIOUS CONCENTRATIONS OF PENICILLIN*

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It is a well-established fact that strains of bacteria resistant to various sulfa drugs, as well as strains resistant to penicillin, may readily be obtained by growing bacteria on media containing increasingly higher concentrations of the respective chemicals. The purpose of this study was to make a quantitative survey of the origin of resistant bacteria, and to clarify the genetic aspect of the mechanism through which resistance is formed. A preliminary report summarizing the results obtained is given below.

Material and Method.—A strain of *Staphylococcus aureus* obtained from the Northern Regional Research Laboratory, Peoria, Illinois, carrying the N.R.R.L. number 313, was used in these experiments. This particular strain is employed by several laboratories for assaying penicillin. Before the experiment was started, a broth culture was prepared with bacteria from a single colony, and from this broth culture three agar slants were inoculated. These three stock cultures were kept in a refrigerator and served daily as the source of inoculum for all experiments. In a long series of experiments conducted over a considerable period of time, this procedure yields material that should be genetically more uniform than if the stock were maintained by consecutive transfers.

Penicillin was taken from a lot of sodium salt of penicillin prepared by E. R. Squibb and Sons, New York, which was packed in ampules containing 25,000 Oxford units each. The material of one ampule was dissolved in 10 cc. of phosphate buffer of pH 6, and kept in the refrigerator as a stock solution containing 2500 Oxford units of penicillin per cc. From this, other stock solutions containing 250 and 25 units per cc. were prepared under sterile conditions. Assays made at intervals indicated that the potency of penicillin in the stock solutions was not affected by storage.

The resistance of the bacteria to penicillin was determined by mixing them with an agar-nutrient medium to which the penicillin solution had been added, and plating the mixture in a Petri dish. Precautions were taken not to have the agar warmer than 45 degrees Centigrade.

In order to have a check of the potency of the penicillin, an assay was made for every experiment by diluting the solution used in that experiment to one Oxford unit per cc. and making a standard Oxford cup test. In this way any appreciable decrease in the potency of the penicillin solution would have been detected.

Reaction of Staphylococcus to Penicillin.—The strain of bacteria used in

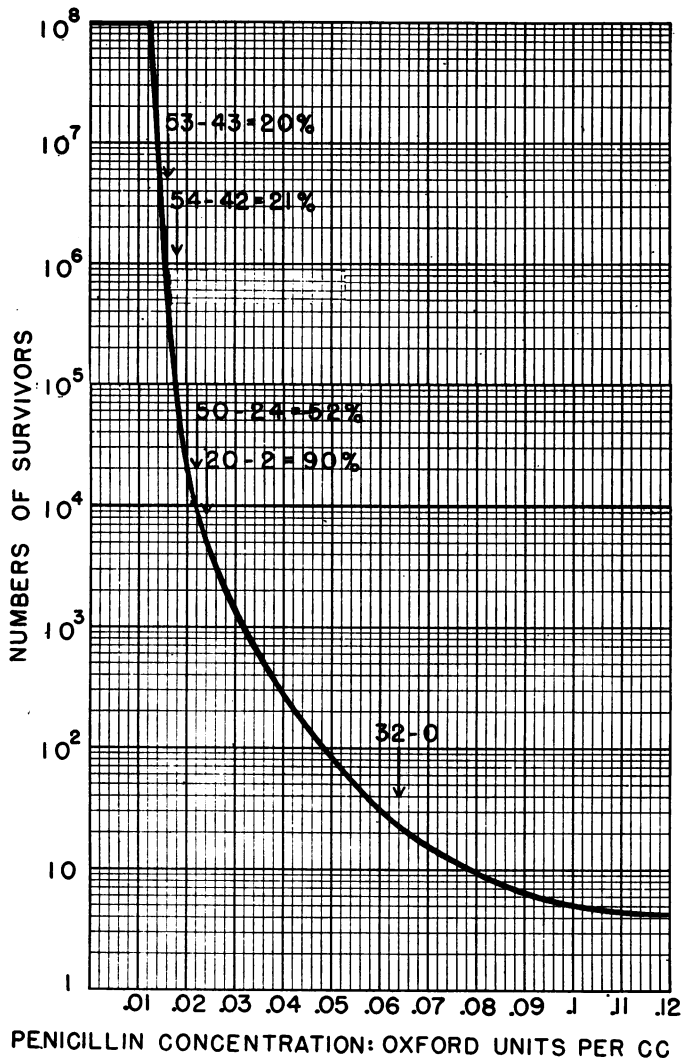

FIGURE 1

Numbers of surviving *Staphylococcus aureus* after plating on nutrient agar containing various concentrations of penicillin. Arrows indicate concentrations at which resistance to penicillin of surviving colonies was tested, and numbers above the arrows show the total number of colonies tested, the number of normal overlaps and the percentage of resistant colonies.

these experiments was affected by various concentrations of penicillin in a manner shown graphically in figure 1. No reduction in colony counts is apparent when the bacteria are plated onto nutrient-agar medium containing

weak concentrations of penicillin, until a threshhold of about 0.012 Oxford units per cc. is reached. A slight increase in concentration after this point produces a striking effect. For example, at a concentration of 0.014 only 10% of colonies appear; at a concentration of 0.016 the survival is 1%; at a concentration of 0.018, 1 per thousand; at a concentration of 0.05, about 1 per million; and a concentration of about 0.15 eliminates all bacteria. Six independent experiments gave very similar results, and the curve shown in figure 1 was fitted to the combined data of these experiments.

Thirty-two strains were established by inoculating broth with bacteria taken from 32 colonies which developed from surviving bacteria plated onto nutrient-agar medium containing 0.064 Oxford units of penicillin per cc. Not more than two colonies from any one plate were used for starting new strains, and the bacteria of the different plates were taken from different cultures. This precaution was taken in order to have strains that were not closely related. All of the 32 strains so established were tested for resistance to various concentrations of penicillin and were found to withstand concentrations considerably higher than the parent strain. The bacteria that formed colonies on plates with the nutrient medium containing 0.064 units per cc. of penicillin did so because they were resistant to at least that concentration of penicillin.

In a similar manner, 20 colonies which had formed on medium containing 0.024 units were tested on various concentrations of penicillin; 18 of them were more resistant than the original strain, while 2 were not. Therefore, at that concentration about 90% of surviving colonies had higher resistance than the parent strain, and 10% were normal overlaps—that is, chance survivors in a survival probability curve.

At a still lower concentration—namely 0.022 units per cc.—there were 50 survivors tested, 24 (or 48%) of which were normal overlaps. At a concentration of 0.018 units, 42 (or about 79%) of the 54 survivors tested were normal overlaps; and at 0.016 units, 43 (or 80%) of the 53 survivors tested were normal overlaps.

From these tests it is evident that at a concentration of 0.064 units per cc. of penicillin all tested survivors had higher resistance than the parent strain, while even at lower concentrations a considerable proportion of survivors were resistant. In figure 1 the arrows indicate concentrations from which survivors were tested, and the numbers above the arrows show the total number of tests, the number of normal overlaps and the percentage of resistant colonies.

Is the Resistance Inherited?—The evidence that has been accumulated suggests an affirmative answer to this question. A resistant strain isolated from the medium containing 0.064 units of penicillin was kept in the refrigerator on an agar slant for three months without any change in the de-

gree of resistance. Ten strains isolated in a similar manner were passed through 20 broth transfers, and tests for resistance were made at the end of that period as well as several times during the process. No change in the degree of resistance was observed. These strains, which were isolated after one passage in penicillin, acquired permanent resistance.

Origin of Resistant Bacteria.—Two alternate mechanisms can be visualized as responsible for the origin of bacteria resistant to certain concentrations of penicillin: (1) Resistance is an acquired characteristic, which develops through interaction between bacteria and penicillin when the two are in contact with each other. (2) Resistance is an inherited characteristic, which originates through mutation and whose origin is independent of penicillin treatment; resistant mutants occur at random, in a small fraction of a population, and, since a certain concentration of penicillin eliminates all non-resistant individuals, the resistant ones are selected out from the population by the treatment.

Which of these two mechanisms is responsible for the origin of resistance can be determined with the aid of a modification of the method developed by Luria and Delbrück[1] in their study of changes in bacteria from bacteriophage-sensitivity to bacteriophage-resistance. In the majority of experiments reported in this paper, bacteria were in contact with penicillin only during the time when the test for resistance was being carried on. Otherwise they were grown in the broth medium free of penicillin. If the resistance is induced through interaction between bacteria and penicillin when they are in contact with each other, it would be expected that approximately similar numbers of resistant bacteria would be obtained when samples containing similar numbers of bacteria are plated onto nutrient agar containing a certain concentration of penicillin, irrespective of the origin of these samples. The situation would be quite different in the event that the origin of resistance is mutational. In such case, one would expect to obtain similar numbers of resistant colonies only in samples taken from the same culture. If, however, each of the samples came from a separate culture, and mutations occur at random, then one would expect to obtain a large number of resistant colonies from cultures in which mutation happened to occur early in the growth of the culture and a small number of resistant colonies from cultures in which mutation happened to occur late, provided resistant bacteria grow more or less like the normal ones. If resistance originates by mutation, then, the variation in number of resistant bacteria between samples taken from separate cultures should be much greater than between samples taken from the same culture.

A critical experiment to distinguish between these two possibilities was planned as follows: A saturated broth culture of bacteria was diluted to 10^{-6}, so that the broth contained about 300 bacteria per cc. 0.3 cc. of this was placed in each of 30 small test tubes. One large test tube contain-

ing about 15 cc. of broth was inoculated with another sample of 0.3 cc. from the same dilution. All 31 test tubes were incubated at 37°C. for 18 hours, and precautions were taken to prevent evaporation from the tubes containing the small cultures. After 18 hours of incubation, 0.7 cc. of broth was added to each of these 30 small tubes, in order to reduce the error when the contents of each tube were taken out and plated. The number of bacteria was determined by sampling 10 of the small tubes, taking 0.05 cc. of material from each and assaying it on nutrient agar after making proper

TABLE 1

NUMBER OF BACTERIA RESISTANT TO CONCENTRATION OF 0.064 OXFORD UNITS OF PENICILLIN PER CC. OF AGAR MEDIUM IN SAMPLES TAKEN FROM A SERIES OF INDEPENDENT CULTURES AND SIMILAR SAMPLES TAKEN FROM A SINGLE CULTURE WHICH ASSAYED 2.3 × 10^8 BACTERIA PER CC.

SAMPLES FROM INDEPENDENT CULTURES					SAMPLES FROM A SINGLE CULTURE	
CULTURE NO.	NO. OF BACTERIA PER CC.	NO. OF RESISTANT BACTERIA	CULTURE NO.	NO. OF RESISTANT BACTERIA	SAMPLE NO.	NO. OF RESISTANT BACTERIA
1	1.83 × 10^8	33	11	196	1	27
2	1.79	18	12	66	2	35
3	1.82	839	13	28	3	34
4	1.79	47	14	17	4	32
5	2.02	13	15	27	5	33
6	2.05	126	16	37	6	27
7	1.76	48	17	126	7	25
8	1.85	80	18	33	8	28
9	2.06	9	19	12	9	34
10	2.02	71	20	44	10	38
			21	28	11	25
			22	67	12	29
			23	730	13	31
			24	168	14	38
			25	44	15	31
			26	50	16	23
			27	583	17	16
			28	23	18	21
			29	17	19	30
			30	24	20	21
Average	1.9 × 10^8	128.4	..	116	..	28.9
Variance	1.35	57255	..	35399	..	39.8
χ^2	7.082	4459	..	6103	..	22.7
P	0.6	.....	..	...	..	0.3

serial dilutions. The number of bacteria in the large culture was determined by a similar assay. The material from the 30 small cultures (containing 0.3 cc. of saturated bacteria to which 0.7 cc. of broth had been added) was plated onto Petri dishes with nutrient agar containing 0.064 Oxford units of penicillin per cc. At the same time, twenty 0.3-cc. samples containing saturated bacteria from the large culture were plated in agar with 0.064 units of penicillin per cc.

Results of this experiment are given in table 1. It may be seen that the growth of bacteria in the different small cultures was fairly uniform, the average titre in ten cultures being 1.9×10^8 of individuals per cc.; and that this was fairly close to the titre of 2.3×10^8 per cc. reached in the large culture. That is, samples taken from the individual cultures and from the large culture contained approximately similar numbers of bacteria. It is evident from the table that the variation between the numbers of resistant bacteria on plates from samples taken from a single culture is small, the extremes being 16 and 38, the variance slightly larger than the average, and the probability that this variation is due to chance 30 per one hundred trials. On the other hand, the variation in number of resistant bacteria among samples taken from independent cultures is considerable, with extremes of 9 and 839 in cultures number 1 to number 10, and 12 and 730 in cultures numbers 11 to 20, with a variance greater than 200 times the average, and an insignificant probability that such a distribution may be due to sampling.

The results of this experiment, therefore, favor the assumption that resistance to certain concentrations of penicillin originates through mutation and that resistant bacteria may be found in any large population. The proportion of resistant bacteria depends on the mutation rate. The experiment was repeated three times, and similar results were obtained.

Another experiment furnished supporting evidence for the conclusion that resistance does not originate as a result of contact between bacteria and penicillin. In a study of the action of penicillin on Staphylococcus, it was found that penicillin affects principally dividing bacteria, while nondividing bacteria can be kept for a considerable length of time in the penicillin-containing medium. To a saturated broth culture, containing 3.7×10^8 bacteria per cc., a sufficient amount of penicillin was added to make the concentration in the medium 25 Oxford units per cc. The culture was kept at 37°C., and after five days an assay showed that it contained 2.3×10^8 living bacteria per cc., while the assay for penicillin indicated that the concentration was not appreciably changed. These bacteria, exposed to 25 units of penicillin for five days, were washed to remove penicillin from the medium and tested for their resistance to various concentrations of this chemical. They were found to be no more resistant to penicillin than bacteria of the original strain. This shows that contact with penicillin does not make resting bacteria resistant.

Degree of Resistance.—In the strain of Staphylococcus used in these experiments, there is about one bacterium per 2×10^8 that survives a concentration of penicillin of 0.125 units per cc. At a concentration of 0.15 units per cc., there are no survivors. However, in strains established from survivors on an 0.064 concentration, there were a few individuals resistant to 0.15 units; in strains developed from survivors on an 0.125 concentration,

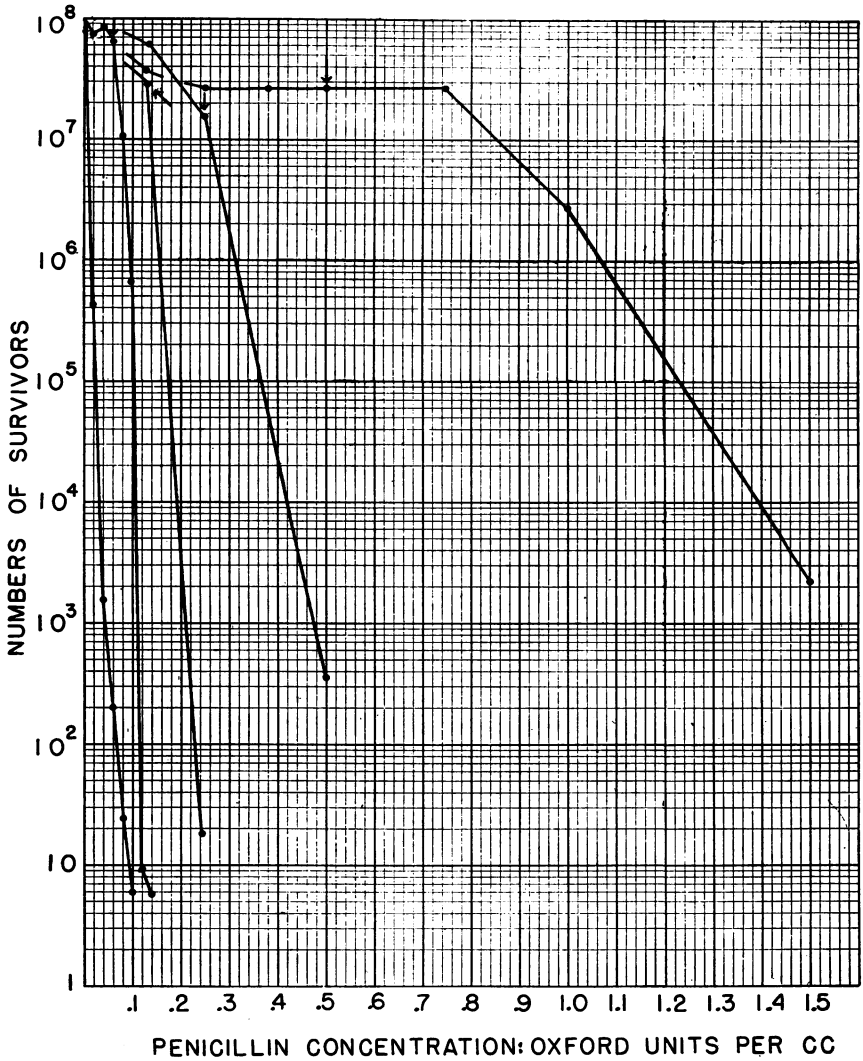

FIGURE 2

Numbers of survivors on various concentrations of penicillin, for the stock strain of *Staphylococcus aureus* and for four resistant strains developed through repeated selection.

there were individuals resistant to 0.25 units; strains from these latter survivors had individuals resistant to 0.5 units; strains from these contained individuals resistant to 4 units; and from these a strain was isolated that was not affected by a concentration of 250 units of penicillin per cc. of the agar medium.

The curves in figure 2 show the survival numbers, with various concentrations of penicillin, for bacteria of the stock strain and of four strains developed by the process of selection described in the previous paragraph. It is evident that the building up of resistance is more rapid with each selection step. That is, a concentration of 0.15 units is sufficient to eliminate all bacteria of the original strain, while to eliminate all bacteria of the first-step resistant strain a concentration of about 0.2 units is required, for the second-step resistant strain about 0.4 units, for the third-step about 1.0 units, and for the fourth-step about 7 units. The fifth-step strain was for all practical purposes completely resistant to penicillin.

As a check against contamination, a bacteriophage strain was isolated which lyses the Staphylococcus strain used in these experiments. This phage lysed also the completely resistant strain mentioned above, and all other resistant strains with which it was tested, indicating that these strains were derived from the original one rather than contaminants.

Discussion.—The evidence reported in this paper makes it probable that resistance of Staphylococcus to certain concentrations of penicillin is not induced by the action of penicillin on bacteria, but arises independently by mutation.

In any large population of bacteria of the strain of Staphylococcus used in these experiments there are some individuals resistant to certain low concentrations of penicillin. If this population is exposed to the action of such concentrations of penicillin, non-resistant individuals are eliminated while the resistant survive. Thus penicillin acts as a selective agent which suppresses non-resistant bacteria.

It is clear from the data that resistance is a complex characteristic, and that it must involve a number of mutations; if it is assumed that genes are responsible for these mutations, a number of genic changes must be involved. Such a situation is not unusual. A close parallel was described by Demerec and Fano[2] in the case of strain B of *Escherichia coli*, where about 20 distinguishable mutant types showing resistance to one or more of the seven phages were detected. Since it would be possible to isolate many more phages affecting the B strain of coli, it is evident that the actual number of mutants affecting resistance is considerably larger than the number detected.

It has been shown that degree of resistance can be increased by selection, and that the building up of resistance is more rapid with each selection step (Fig. 2). This can readily be explained by the mutation hypothesis. It is assumed that there are a number of genes that affect resistance to penicillin, and if any one of them mutates, the individual in which such mutation occurs acquires resistance to a certain concentration of penicillin. Mutants may differ in degree of resistance, but the resistance of individuals in which only one gene has mutated (single mutants) is never very high.

In a single-mutant strain, mutations may occur in other genes for resistance; and when two mutant genes are together in one individual (double mutants) their effect is cumulative. Moreover, it happens that the resistance of a double mutant is higher than the sum of resistances of two single mutants. If a third gene for resistance, a fourth, etc., mutate in the same line, the combined effect of all these mutations is a high degree of resistance or complete resistance.

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Summary.—In experiments with *Staphylococcus aureus*, strains resistant to penicillin were developed, which retained the property of resistance during the period covered by the experiments. Evidence is presented indicating that resistance is not induced by the action of penicillin on bacteria, but originates through mutation, and that penicillin acts as a selective agent to eliminate nonresistant individuals. Degree of resistance can be increased by exposure to higher concentrations of penicillin, and this increase is interpreted as due to summation of the effects of several independent genetic factors for resistance which undergo consecutive mutation.

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[1] Luria, S. E., and Delbrück, M., *Genetics* **28**, 491–511 (1943).

[2] Demerec, M., and Fano, U., *Genetics* **30**, (in press) (1945).

THE LAW OF MASS ACTION IN EPIDEMIOLOGY

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Almost all workers in the analytical theory of epidemics assume that the rate at which an infection passes in a population is proportional jointly to the product of the number of persons I who are infectious and the number of persons S who are susceptible to the infection.[1–6] This is called the law of mass action. Thus if the rate of new infections be C the law is written as

$$C = r I S, \tag{1}$$